ORIGINAL RESEARCH

Protective effects of caffeic acid phenethyl ester on radiation induced lung injury in rats

Oguz Galip Yildiz MD¹

Serdar Soyuer MD¹

Recep Saraymen PhD²

Celalettin Eroglu MD¹

Departments of Radiation Oncology,¹ Biochemistry,²
Erciyes University, Faculty of Medicine,
Kayseri, Turkey

Manuscript submitted 11th May, 2008

Manuscript accepted 12th August, 2008

Clin Invest Med 2008; 31 (5): E242-E247.

Abstract

Purpose: The prevention of radiation-induced pulmonary toxicity may help to improve radiation therapy in the cancer patient. The aim of this study was to investigate the pulmonary protective effects of caffeic acid phenethyl ester (CAPE), an antioxidant, on radiation-induced lung injury in rats.

Methods: 30 Wistar albino rats were divided into three groups and treated with saline, Radiation (RT) and RT + CAPE respectively. All rats were treated with CAPE (50 µmol/kg i.p.) or saline. The first dose of CAPE was injected 24 h before radiation and application continued daily, with radiation in second day and 2 days more after the radiation treatment. Radiation dose was 800 cGy for total body. At 72 hr after the last radiation application, under general anesthesia using *ip* ketamine, the lungs were removed immediately after decapitation. After sacrifice, antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) activities and malondialdehyde (MDA) levels were evaluated in lung tissue.

Results: The level of malondialdehyde (MDA) was higher in the RT group (233.4±1.5 nmol/g protein) than in both the control (131.8±0.92) and the RT + CAPE (151.4±1.8) groups ($P < 0.001$). However, CAT activity was decreased in the RT group (7.26±0.27 U/mg protein) compared with control (8.49±0.51) and increased again in the RT + CAPE group (8.31±0.56; $P < 0.001$). In accord with CAT activity, SOD activity in the RT group (0.42±0.07 nmolMDA/g wet tissue) was different from the control (0.78±0.02) and RT + CAPE (0.86±0.06) groups ($P < 0.001$).

Conclusion: CAPE application with radiation therapy attenuated radiation induced pulmonary injury *in vivo*, possibly by its antioxidant effect.

When radiation is absorbed by a biological material, free oxygen radicals are produced. The radicals are highly reactive molecules and, via chemical bonds that produce chemical changes, initiate a chain of events that result in biological damage. They can be produced either directly in the target molecule (usually DNA) or indirectly in other cellular molecules and they diffuse far enough to reach and damage critical targets. Most indirect effects occur by free radicals produced in water, since water makes up 70-80 % of mammalian cells.¹ In this way, chemotherapy and radiotherapy eliminate cancer cells, but their nonspecific targeting also destroys normal, healthy cells, particularly in epithelial tissues. Damage to the epithelium of the respiratory tract results in a pathologic condition known as pneumonitis.²

Radiation (RT) is frequently used to treat tumors in and around the thorax. In these patients, RT-induced lung injury is common, occurring in 5% to 20% of patients with lung cancer. The incidence is somewhat less, 5% to 15%, for patients with tumors such as mediastinal lymphoma and breast cancer.³

Radiation pneumonitis (RP) is a major dose-limiting toxicity in thoracic irradiation and usually occurs within 6 months of therapy and can manifest as cough, dyspnea, fever, malaise, or hypoxia in severe cases. Treatment might require considerable supportive measures, including steroids, oxygen supplementation, or even mechanical ventilation. Even with treatment, RP remains potentially fatal.⁴ Although the pathophysiology of pulmonary fibrosis (as a component of RP) has not been well clarified, it has generally been hypothesized that activated inflammatory cells which accumulate in the lower airways, release harmful amounts of reactive oxygen species (ROS) that result in lung injury, and proliferation of fibroblasts in alveolar walls. The activated fibroblasts produce increased amounts of extracellular matrix proteins that distort the normal lung architecture and impair the vital gas exchange function of the lungs.⁵

Caffeic acid phenethyl ester (CAPE) is an active component of propolis, which is a resinous hive product collected by honeybees from various plant sources.⁶ CAPE has been exhibits anticancer, anti-inflammatory and immunomodulatory activities in a broad spectrum of systems.^{7,8} 10 mol/L, CAPE completely blocks the generation of reactive oxygen species in human neutrophils and the xanthine/xanthine oxidase system.⁹

Lipid peroxidation is a well-established mechanism of cellular injury in humans, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and can decompose to form a complex series of compounds. These include reactive carbonyl compound, which is the most abundant malondialdehyde (MDA). Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation. Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both humans and model systems.¹⁰

Cells continuously produce free radicals and reactive oxygen species (ROS) as part of their metabolic processes. These free radicals are neutralized by an

elaborate antioxidant defense system consisting of enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) as well as numerous non-enzymatic antioxidants.¹¹ This study was designed to determine the possible protective effect of CAPE application against oxidative damage in the lung, induced by radiation using an animal model. Therefore, to determine the efficacy of CAPE in lung tissue, CAT and SOD activities as well as MDA level were measured on the third postburn day.

Materials and Methods

Animals

Thirty in-bred adult male Swiss Albino rats were obtained from the Test Animals Brooding Center of Er-ciyes University. They were fed with standard feed and water under sterile hygienic condition. They were 12 weeks old and weighed 250 $\pm$ 15 g. The light and dark cycle was automatically regulated at 12 hours.

Application categories

The animals were divided into three groups. Each consisted of 10 animals. The first, the control category (C), received only saline as equal in volume to the amount of CAPE administration intraperitoneally (n=10). The second received radiation alone (RT). They were treated with 800 cGy radiation to total body (n=10) in one fraction. The third and final category, the RT + CAPE category, and they were administered 50 μ mol/kg of CAPE intraperitoneally. It is important to emphasise that the third treatment category, received RT + CAPE in the same dose of radiation and CAPE treatment categories (n=10).

External irradiation to total body was given with a special 30x30x5 cm animal-fixing box using gamma rays from the Co⁶⁰ teletherapy machine (Theratron 780 C) with parallel-opposed field at the mean dose rate 3.13 cGy/MU. Radiation for the total body was 800 cGy. An animal fixing box contained five rats for

each irradiation. Dose was calculated as $D_{\max}$ dose at 2.5 cm depth for SSD 80 cm.

Application of CAPE

The CAPE was synthesized by the standard method of Grunberger (7) and administered intraperitoneally once a day at a dose of $50 \mu\text{mol/kg}$. The first dose of CAPE was injected 24 h prior to radiation and continued daily application with radiation in second day and 2 more days after the radiation application. CAPE (Sigma-Aldrich Chemie GmbH, Steinheim, Germany 3 mg/kg of the salt) was dissolved in ethanol and diluted in saline (0.09% NaCl, w/v) to give final concentration of 1%. All experiments were performed in accordance with the guidelines for Animal Research from the National Institutes of Health and were approved by the Committee on Animal Research at Erciyes University, Kayseri/Turkey.

Determination of CAT activity

CAT activity was determined according to Aebi's method.¹² The principle is based on the determination of the rate constant (k ; / s) or the H_2O_2 decomposition rate at 240 nm. The results were expressed as k (rate constant) per gram protein.

Determination of SOD activity

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al.¹³ The principle is based on inhibition of Nitro Blue

Tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the lysate after 1.0 ml of ethanol–chloroform mixture (5:3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as U mg/ protein.

Analysis of lipid peroxidase level

At 72 h after the last treatment, the rat lungs were promptly removed after decapitation under general anesthesia with *ip* ketamine (50 mg/kg). They were weighed and chilled in ice-cold 0.9 % NaCl. After washing with 0.9 % NaCl, tissue homogenates were prepared in 6 ml 1.15 % KCl buffer solution using Edmund Buhler 7400 Tübingen HO4 homogenizer. Kohn and Liversedge described the colorimetric reaction of thiobarbituric acid (TBA) with an unknown substance formed during the aerobic incubation of tissue homogenates. This was later identified by Patton and Kurtz as MDA, a secondary product of lipid peroxidation; the reaction of lipid peroxides with TBA has been widely adopted as a sensitive assay method for lipid peroxidation in animal tissue.¹⁴

The reaction mixture contained 0.2 ml sample, 0.2 ml 8.1 % sodium dodecyl sulfate (SDS), 1.5 ml 20 % acetic acid solution (pH: 5.5) and 1.5 ml 0.8 % aqueous solution of TBA. The mixture was made up to 4.0

TABLE: CAT, SOD activities and MDA and level in C and RT and RT +CAPE groups.

	CAT Umg protein $\pm$ SD	SOD nmolMDA/g wet tissue $\pm$ SD	MDA nmol/g protein $\pm$ SD
1. Control (n=10)	8.492 $\pm$ 0.516	0.781 $\pm$ 0.021	131.78 $\pm$ 0.92
2. RT (n=10)	7.260 $\pm$ 0.268	0.415 $\pm$ 0.066	233.40 $\pm$ 1.53
3. RT+CAPE (n=10)	8.306 $\pm$ 0.575	0.861 $\pm$.064	151.38 $\pm$ 1.79
<i>P</i>			
1-2	0.001	0.001	0.001
1-3	0.892	0.045	0.001
2-3	0.002	0.001	0.001

*RT:Radiotherapy

**CAPE: Caffeic acid phenethyl ester

ml with distilled water and heated at 95 °C for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml mixture of n-butanol and pyridine (15:1 v/v) was added and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbency of the layer (upper layer) was measured at 532 nm by spectrophotometer. Protein was determined by the biuret method. The level of lipid peroxides was expressed in terms of nmol MDA/ g wet-tissue, which was calculated from the absorbance at 532 nm.

Determination of protein content

Protein measurements were made at all stages according to Lowry's method.¹⁵

Statistical analysis

Data were analysed using a commercially available statistics software package (SPSS® for Windows v. 13.0, Chicago, USA). All groups showed normal distribution, so parametric statistical methods were used to analyse the data. A one-way ANOVA test was performed and post hoc Tukey HSD multiple comparisons, were made using least-squares differences. Comparisons to control group was performed with Dunnett test. Results are presented as mean $\pm$ standard deviation (SD); $P < 0.05$ was regarded as statistically significant.

Results

CAT activities were decreased in the RT group compared with C and increased again in the RT + CAPE group ($P < 0.001$): CAT activities were not different between C and RT + CAPE groups. In addition, SOD activity in the RT group was different from both the C and RT + CAPE groups ($P < 0.001$).

The level of MDA was higher in lung tissue homogenate of the RT group than both the C and RT + CAPE groups ($P < 0.001$). There was also a difference in MDA levels between C and RT + CAPE groups ($P < 0.001$).

Discussion

In our work, CAT activities were decreased in the RT group in comparison with control and increased again in the RT + CAPE group. However, CAT activities were not different between the C and RT + CAPE groups. In addition, SOD activity in the RT group was different compared with both C, and RT + CAPE groups. MDA level was higher in lung tissue homogenate of the RT group than in both C and RT + CAPE groups. There was also a difference in MDA levels between C and RT + CAPE groups. All these results indicate that CAPE application with radiation therapy attenuated radiation induced pulmonary injury, possibly by its *in vivo* antioxidant effects.

Cellular biomolecules are altered directly by radiation or damaged indirectly by free radical production.¹ Reactive oxygen species such as superoxide radical, hydroxyl radical and hydrogen peroxide can induce cellular injury through lipid peroxidation reaction in cell membranes.¹¹ Damage with free radicals, particularly the activated oxygen derivatives is the important mechanism in the cell injury. Cell edema, reversible, and cell necrosis, irreversible, cell injuries are caused by free oxygen radicals. With the advent of oxygen radicals, damage to tissue molecules including nucleic acids, membrane lipids, enzymes and receptors entail lipid peroxidation and, in turn, membrane liquidity impairment and cell lysis. As lipid peroxidation is the main pathway for tissue radical damage, blocking of this pathway appears is an attractive strategy to protect the lung from reactive oxygen–nitrogen species-mediated damage.⁹⁻¹¹

Many radioprotective agents have been tried to protect against the deleterious effects of ionized radiation. The most important are sulphhydryl compounds such as cysteine.¹⁶ Vitamin E therapy decreases the radiation injury by its antioxidant effects by preventing free radicals.¹⁷ Walden et al.¹⁸ demonstrated the protective effects of Prostaglandin E₂ in whole body irradiated rats. CAPE is an active component of propolis, which is a popular folk medicine in various

countries possessing a broad spectrum of biological activities, including anticancer, antioxidant, anti-inflammatory, antibiotic, antifungal and antihepatotoxic effects.⁶ CAPE exhibits anticancer, anti-inflammatory and immunomodulatory activities in a broad spectrum of systems.^{7,8} Recently, CAPE was investigated intensively in a number of animal models and various organs, including kidney, intestine, testis, spinal cord, hindlimb and lung.^{7-8,19,20} It has been suggested that CAPE has protective effects against oxidative damage, which has been linked to its free radical-scavenging ability and anti-oxidant capacity.²⁰⁻²⁴ Also, CAPE suppresses lipid peroxidation and inhibits lipoxygenase activity.^{9,23,24}

Pulmonary injury of radiation is well known and is accepted as a dose limiting factor in the treatment of the thoracic malignancy, such as heart, esophagus and breast cancer.²⁵⁻²⁷ Radiation pneumonitis (RP) due to thoracic irradiation usually occurs within 6 months of therapy, which might require considerable supportive measures, including steroids, oxygen supplementation, or even mechanical ventilation. Even with treatment, RP is potentially fatal.² Chen et al.²⁸ reported that CAPE decreases acute pneumonitis after irradiation *in vitro* and *in vivo*. CAPE decreases the cascade of inflammatory responses induced by thoracic irradiation without causing injury in normal lung tissue. This provides a rationale for combining CAPE and thoracic radiotherapy for lung cancer treatment in further clinical studies.

The possible radioprotective effects of CAPE against radiation induced pulmonary injury were tried to be investigated in this study. We found that the radiation caused accelerated lipid peroxidation in the lung tissue of rats, as measured by MDA. It also caused CAT and SOD activity depletion. However, CAPE administration in the irradiated group had reduced MDA value. This indicates that administration of CAPE protected lung effectively from radiation-induced lung injury by an unknown mechanism. These findings suggest that CAPE is important in pro-

tecting the lung tissue from radiation -induced damage in rats *in vivo*.

Conclusions

CAPE may be used to prevent lung injury during radiation therapy as a therapeutic agent for malignant tumors. However, it remains to be determined whether CAPE has a protective role in the histopathological changes due to radiation-induced pulmonary injury. Further investigation is required, with both biochemical and histopathological parameters measured simultaneously.

References

1. Steel GG. Basic Clinical Radiobiology. 2nd edition, Oxford University press;1997.p 133.
2. Borges L, Rex KL, Chen JN. A protective role for keratinocyte growth factor in a murine model of chemotherapy and radiotherapy-induced mucositis. *Int J Radiat Oncol Biol Phys* 2006; 66:254–62.
3. Marks LB, Yu X, Vujaskovic Z et al. Radiation-induced lung injury Semin in Radiat Oncol 2003;13:333-45
4. Hope AJ, Lindsay PE, EI Naqa I et al. Modeling radiation pneumonitis risk with clinical, dosimetric, and spatial parameters. *Int J Radiat Oncol Biol Phys* 2006;65:112–24
5. Weissler JC. Idiopathic pulmonary fibrosis: cellular and molecular pathogenesis. *Am J Med Sci* 1989;297:92–104.
6. Banskota AH, Tezuka Y, Kadota S. Recent progression pharmacological research of propolis. *Phytother*. 2001;15:561–71.
7. Grunberger D, Banerjee R, Eisinger K et al. Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. *Experientia* 1988;44:230–2.
8. Natarajan K, Singh S, Burke TR et al. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF- κ B. *Proc Natl Acad Sci USA* 1996;93:9090–5.
9. Sud'ina GF, Mirzoeva OK, Pushkareva MA et al. Caffeic acid phenethyl ester as alipoxygenase inhibitor with antioxidant properties. *FEBS Lett* 1993;329:21–4.

11. Draper HH, Hadley M. A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. *Xenobiotica* 1990;20:901-7.
12. Djordjevic VB: Free radicals in cell biology. *Int Rev Cytol* 2004;237: 57-89
13. Aebi H. Catalase. In: *Methods of Enzymatic Analysis*, Bergmeyer HU (ed.). Academic Press: New York; 1974;673-7.
14. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. *Clin Chem* 1988 ;34:497-500.
15. Torre PD, Imondi AR, Bernardi C et al. Cardioprotection by Dexrazoxane in rats treated with doxorubicin and paclitaxel. *Cancer Chemother Pharmacol* 1999;44:138-42.
16. Lowry O, Rosenbraugh N, Farr L et al. Protein measurement with the Folin-phenol reagent. *J Biol Chem* 1951;183:265-75
17. Patt HM, Turee EB, Straube RL et al. Cysteine protection against X irradiation. *Science* 1949 ;110:213-4.
18. Sarma L, Kesavan PC. Protective effects of vitamins C and E against gamma ray induced chromosomal damage in mouse. *Int. J Radiat Biol* 1993; 63:759-64.
19. Walden TL, Patchen M, Snyder SL et al. 16 Dimethyl prostoglandin E2 increases survival in mice following irradiation. *Radiat Res* 1987;109:440-8
20. Gurel A, Armutcu F, Sahin S et al. Protective role of α -tocopherol and caffeic acid phenethyl ester on ischemia-reperfusion injury via nitric oxide and myeloperoxidase in rat kidneys. *Clin Chim Acta* 2004;339:33-41.
21. Koltuksuz U, Ozen S, Uz E et al. Caffeic acid phenethyl ester prevents intestinal reperfusion injury in rats. *J Pediatr Surg* 1999;34:1458-62.
22. Uz E, Sogut S, Sahin S et al. The protective role of caffeic acid phenethyl ester (CAPE) on testicular tissue after testicular torsion and detorsion. *World J Urol* 2002;20:264-70.
23. Ilhan A, Koltuksuz U, Ozen S et al. The effects of caffeic acid phenethyl ester (CAPE) on spinal cord ischemia/reperfusion injury in rabbits. *Eur J Cardiothorac Surg* 1999;16:458-63.
24. Calikoglu M, Tamer L, Sucu N et al. The effects of caffeic acid phenethyl ester on tissue damage in lung after hindlimb ischemia-reperfusion. *Pharmacol Res* 2003;48:397-403.
25. Ozyurt H, Sogut S, Yildirim Z et al. Inhibitory effect of caffeic acid phenethyl ester on bleomycin-induced lung fibrosis in rats. *Clin Chim Acta* 2004;339:65-75.
26. Shulman LN, Braunwold E. Cardiac effects of radiation therapy and chemotherapy. In Braunwold E. *Heart Disease : A textbook of cardiovascular medicine*. 5th edition. WB Saunders Company 1997. p.1799-804.
27. Vasigara SW, Subramaniam S, Shyama S, et al. Changes in erythrocyte membrane lipids in breast cancer after radiotherapy and chemotherapy. *Chemotherapy* 1996;42:65-70.
28. Mukherjee S, Aston D, Minett M, et al. The significance of cardiac doses received during chemoradiation of oesophageal and gastro-oesophageal junctional cancers. *Clin Oncol* 2003;15:115-120.
29. Chen MF, Keng PC, Lin PY, Yang CT, Liao SK, Chen WC. Caffeic acid phenethyl ester decreases acute pneumonitis after irradiation in vitro and in vivo. *BMC Cancer* 2005;9:158.

Correspondence to:

Oguz Galip Yildiz MD
Departments of Radiation Oncology
Erciyes University, Faculty of Medicine,
38039 Kayseri /Turkey
e-mail: yildizog2003@yahoo.com